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# Memory Dysfunctions: An Integration of Animal and Human Research from Preclinical and Clinical Perspectives[a]

*Editors*

DAVID S. OLTON, ELKAN GAMZU, and SUZANNE CORKIN

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# EXHIBIT 22

Alzheimer Disease. From Molecular Biology to Therapy
edited by R. Becker and E. Giacobini


# CHOLINESTERASE INHIBITORS DO MORE THAN INHIBIT CHOLINESTERASE

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## INTRODUCTION

The cholinergic system plays an important role in learning and memory processes. The crucial role of acetylcholine (ACh) is supported by three lines of evidence. The first line is the effects of pharmacological manipulation using agonists or antagonists at both nicotinic and muscarinic receptors (Decker and McGaugh, 1991; Murray and Fibiger, 1985; Mandel and Thal, 1988; Mandel et al., 1989; Drachman and Leavitt, 1974; Drachman, 1982; Flicker et al., 1992; Vanderwolf et al., 1990; Wesnes et al., 1990). Second is the fact that adverse effects of lesioning cholinergic nuclei are ameliorated by intracerebral transplantation of fetal cholinergic cells or genetically modified tissue (Alkon et al., 1991; Dekker et al., 1991; Page et al., 1991; Berger-Sweeney et al., 1994; Dunnett et al., 1985; Gage and Bjorklund, 1986; Nilsson et al., 1987). The third is the fact that deficits in cholinergic cortical innervation and decreases in nicotinic receptors are seen in humans during aging and Alzheimer's disease (AD) (Perry et al., 1978; Whitehouse et al., 1982; Bartus et al., 1982; Giacobini et al., 1989; Schröder et al., 1995). Results from these investigations are consistent with the concept that cholinergic function is required for learning and memory. A recent article of Winkler et al. (1995) demonstrates that cerebral ACh is not only necessary for cognitive behavior in the rat but its presence and function within the neocortex is also sufficient to improve learning deficits and restore memory in experimental animals following severe damage to the nucleus basalis of Meynert. By analogy, in Alzheimer patients, restoration of cholinergic neurotransmission should be sufficient to ameliorate impaired learning and memory. The formulation of this hypothesis has been followed by numerous clinical trials using various types of cholinergic drugs (Giacobini, 1994). This

chapter will focus mainly on cholinesterase inhibitors and on effects of these drugs other than cholinesterase inhibition in CNS.

| COMPOUND | COMPANY | CHARACTERISTICS | PHASE* |
|---|---|---|---|
| YM 796 | Yamanouchi | $M_3$ weak agonist<br>$M_1$ selective agonist | II Japan |
| RS 86 | Sandoz | $M_1$ agonist | exper. |
| AF-102B<br>FKS-508 | Snowbrand<br>Israel I.B.R. - Forest | $M_1$ ($M_3$)agonist | II-III<br>Japan US |
| BIBN-99 | K. Thomae GmbH.<br>Boehringer | $M_2$<br>antagonist | exper. |
| CI-979/RU35926<br>Milameline | Warner Lambert<br>Roussel | Partial $M_1$<br>agonist (non selective) | II |
| LY287041 | Eli Lilly | $M_1$ agonist | exper |
| SR-46559 | Sanofi | $M_1$ agonist. (not sel.)<br>$M_2$ antagonist | exper |
| CI-1002 | Parke Davis | AChE inhibitor<br>$M_1$ antagonist | exper. |
| PD-151832 | Parke Davis | $M_1$ agonist | exper. |
| PD-142505 | Parke Davis | $M_1$>$M_2$ agonist | exper. |
| LY246708<br>Xanomeline | Lilly/Novo-<br>Nordisk | $M_1$ agonist | II-III |
| SB202026 | Smith Kline Beecham | $M_1$ agonist | I-II Eur |
| PDC 008.004 | Pharm Disc Corp. | $M_2$ antagonist | exper |
| RO 46-5934 | Hoffman La Roche | AChE inhibitor<br>$M_2$ antagonist | exper. |

TABLE I Muscarinic agonists and antagonists of clinical interest *Clinical Phase in USA; Eur = Clinical Phase in Europe

*Cholinergic Therapy: Which way to go? Muscarinic agonists and antagonists*

For as long as development of ChEI has been in progress, a parallel line of research has attempted to develop muscarinic drugs such as agonists to stimulate selectively postsynaptic $M_1$ receptors or antagonists to inhibit the effect of $M_2$ presynaptic receptors in order to improve ACh release in brain. Neither approach has produced a highly selective drug; therefore, many compounds have never reached clinical trials or are still at early stages (Table I). One major obstacle continues to be the presence of severe side effects, particularly of gastro-intestinal and cardiac nature. Because of the present limitations, future drugs need to demonstrate higher receptor selectivity. The number of muscarinic agonists presently in clinical trial is lower than that of ChEI (Table I). Data from clinical trials with muscarinic agonists are still scanty, particularly if compared to the rich literature about ChEI Some differences are starting to emerge between the clinical potential of these two classes of drugs. Cholinesterase inhibitors seem to exert a predominantly cognitive effect (attention, memory, concentration) while muscarinic agonists seem to act mainly on behavioral aspects of the diseases (Table II). If confirmed, combination of properties of both agents may prove to be of benefit (Table VI) Also, there may be differences in side effects (Table II). For muscarinic agonists, the main obstacle is still to overcome autonomic side effects which seem to be substantial even in the lately developed products.

*Nicotinic Agonists*

Reduction in nicotinic acetylcholine receptor (nAChR) pharmacology and expression have been among the first reported neurochemical landmarks of AD (cf. DeSarno et al., 1982; Giacobini et al., 1989; Schröder et al., 1995). Therapeutic strategies based on the findings of impaired nicotinic cholinergic transmission are being developed aimed at stimulating decreased nAChR function ( Schröder et al., 1995). Studies with microdialysis have shown that nicotine as well as several analogues investigated in our laboratory may stimulate the release of norepinephrine, dopamine and serotonin together with ACh (Summers et al., 1994; Summers and Giacobini, 1995). The different effects of various nicotinic agonists on cortical neurotransmitters suggest a

| CLASS | CLINICAL EFFECTS | SIDE EFFECTS |
|---|---|---|
| Cholinesterase inhibitors | Predominantly cognitive | very low incidence with new drugs |
| Muscarinic agonists | Predominantly behavioral | significant cholinergic side effects |

TABLE II. Differences in clinical potential and side effects between cholinesterase inhibitors and muscarinic agonists

| Compound | Country | Company | Clinical Phase**** | Side Effects Comments |
|---|---|---|---|---|
| Physostigmine slow release | USA | Forest | III | N.A. |
| ENA 713 | USA/Europe | Sandoz | III | Low side effects |
| Eptastigmine | USA/Italy | Mediolanum | III | Low side effects |
| E-2020 | USA/Japan | Eisai | III | Low side effects |
| MDL 73,745 | USA/Europe | Marion Merrell Dow | II | Low side effects |
| Metrifonate | USA/Germany | Bayer/Miles | III | Low side effects |
| Tacrine (THA) * | USA/Europe | Warner-Lambert | IV | Hepatotoxicity |
| Velnacrine (HP029)** | USA/Europe | Hoechst-Roussel | II | Hematology *** Hepatotoxicity |
| Suronacrine (HP128)** | | | | |
| Galanthamine | Germany | Shire Pharm. | II | Low side effects |
| | USA | Ciba-Geigy | | |
| Huperzine A | China | Chinese Acad. Sci. | III | N.A |
| NX-066 | England/USA | Astra Arcus | II | N A |
| CP-118,954 | USA | Pfizer | II | N.A. |
| KA-672 | Germany | Schwabe | I | N A. |
| NIK 247 | Japan | Nikken | III | Low side effects |
| TAK 147 | Japan | Takeda | III | Low side effects |

TABLE III Cholinesterase inhibitors AD clinical trials (1996). * other indications: HIV, tardive dyskinesia; ** withdrawn; ***neutropenia or agranulocytosis ****Unless Phase in USA. N.A. = data not available

differential action on subtypes of receptors and specific pre- and post-synaptic interactions (Summers et al., 1995). A second approach to nicotinic cholinergic therapy of AD has been the development of cholinergic channel activators such as ABT-418 (Arneric et al., 1994). This compound is undergoing clinical studies.

*Cholinesterase Inhibitors*

ChEI are, so far, the only drugs demonstrating clinical efficacy in the treatment of AD (c.f. Giacobini, 1994, 1995). The principle used behind indirect cholinomimetic therapy with ChEI is to reduce ACh hydrolysis in central nervous system (CNS) nerve terminals by means of ChEI (Becker et al., 1991). The resulting increase in extracellular ACh concentration should restore central cholinergic hypofunction and improve memory and cognition (Becker and Giacobini, 1988). The use of a ChEI (THA, tacrine, tetrahydroaminoacridine) has resulted in a dose-dependent clinical efficacy in 20-30% of AD patients (Knapp et al., 1994). Since 1988, the number of ChEI in development for AD treatment has increased from 6 to 13 in 1996 (Table III). In spite of this fact, up to 1995 tacrine has been the only drug approved for the indication of AD, both in the USA and Europe (France, Sweden, Italy, Finland, Switzerland).

A 1996 list of ChEI (Table III) in clinical trials includes at least 13 drugs, most of which have already advanced to clinical phase III. The next two-year period (1996-1998) should be the most crucial in this process of selection. The use of tacrine in several thousand patients in Europe and USA has taught a precious lesson (Table IV). Drug companies and research laboratories have profited both pre-clinically and clinically from this experience. The new generation ChEI to replace tacrine in the market will have to fulfill certain requirements which are listed in Table V. It remains to be demonstrated whether or not such a drug(s) is(are) already present among the dozen in clinical trials (Table III).

The major focus in developing a successor to tacrine is obviously avoiding toxicity including liver, bone marrow and CNS effects. In order to benefit the patient, help caregivers and convince skeptical physicians of a real gain, the therapeutical effect should be extended to at least half of the patients and should be maintained for a period of at least 2-3 years. It is also important that the improvement seen in cognitive performance translates into a significant enhancement of activities of daily living and in a demonstrable delay in institutionalization. The goal of slowing down deterioration is clearly in the mind of researchers. To test this effect, we are still missing crucial experimental models and selective clinical markers. In addition, clinical assessment tools are not perfected enough to measure a neuroprotective effect. Consequently, a drug with genuine neuroprotective effects may well not be recognized as such in clinical trials. Another problem with ChEI is the identification of those patients most likely to benefit from therapy. Choosing

the stage of disease at which to start medication may also be crucial for the success of the future ChEI.

*Combinations of ChEI with muscarinic agonists or antagonists to obtain potentiating effects*

Using microdialysis, we observed that in rat cortex the extracellular concentration of ACh following AChE inhibition is regulated through muscarinic receptors (Messamore et al., 1993). These data suggest that a combination of an AChE inhibitor and a presynaptically acting selective muscarinic antagonist could represent a useful strategy to: 1) enhance the release of ACh, and 2) simultaneously elevate its extracellular concentration. Based on this principle, various combinations of ChEI and muscarinic acting drugs or a new drug combining both actions can be suggested (Table VI). Some of these approaches are being attempted (Table I) using either agonists or antagonists. Also interesting would be to explore the combination of an M2 selective antagonist with a ChEI to augment ACh release (Tables I and VI). Other responses may also become attenuated. Tolerance, or a kind of "wearing off" phenomenon, to the clinical effect could develop as a result of receptor desensitization or down-regulation following a prolonged ChEI treatment. Modulation of ACh release, up-regulation of nicotinic and down-regulation of muscarinic receptors have been reported in the CNS of rats following prolonged administration of physostigmine (PHY) (De Sarno and Giacobini, 1989). Therefore, it seems useful to test, experimentally and clinically, various combinations which may prevent or reduce tolerance to drug effect. It is probable that high doses of ChEI would cause tachyphylaxis and enhanced side effects. This will make it necessary to individualize doses for each patient and use an appropriate mode of administration.

1. Low toxicity levels of a ChEI can be tolerated provided there is no fatal outcome (side effects/benefit ratio).
2. A modest improvement in the patient may be seen as a significant advantage from the caregiver's point of view.
3. The patient can be taken off tacrine and then be put back again without totally losing efficacy of the drug.
4. Tacrine should not be discontinued abruptly (high risk for withdrawal with psychotic symptoms).
5. There is a high individual variability in size of effective dose and in occurrence of side effects.
6. Estrogens may have a synergistic effect with tacrine.
7. The cost of the drug should be such to make it accessible to a vast number of patients, including those who are not insured (pharmaco-economic question).

TABLE IV What did we learn from the tacrine experience?

| Compared to Tacrine | General Prerequisites |
|---|---|
| Be less toxic | Slow down deterioration |
| Show stronger clinical efficacy | Improve performance (ADL) |
| Benefit more than 25% of patients | Delay institutionalization Be sold at a moderate price for long-term treatment (5-10 yrs) |

TABLE V. Prerequisites for a new cholinesterase inhibitor to replace tacrine.

*Methodological advances in the study of ChEI pharmacology*

The study of the cholinergic system has been made possible through the development of sensitive micromethods during the last forty years. Lately, a sensitivity for ACh determinations in the low femtomole (fmole) range was reached using highly sensitive electrochemical detectors (ECD) (Table VII).

Based on observations in animals we postulated that ChE inhibition in plasma, erythrocytes or in brain could not be considered as an accurate predictor of changes in cortical ACh (Messamore et al., 1993). Therefore, it became important, following administration of a ChEI, to measure directly CNS ACh levels. This measurement allows one to evaluate the potential of the drug to elevate the neurotransmitter to therapeutically relevant concentrations. Microdialysis *in vivo* is the only method which allows one to carry on such measurements in the awake animal without interference of anesthesia. In particular, when studying the effect of a ChEI it is important to avoid the interaction with a second ChEI. We developed a microdialysis technique which allows fmol range measurement of ACh without introduction of a second ChEI in the probe to artificially magnify ACh levels (Messamore et al., 1993; Cuadra et al., 1994). This technique has been used extensively in our laboratory to examine the effect of several ChEI being tested in clinical trials or to develop novel compounds.

*Cholinesterase inhibitors effect on extracellular concentrations of cortical neurotransmitters*

Clinical and experimental evidence indicates involvement and interactions between the cholinergic system and the biogenic amine systems in the

| COMPOUND TO BE COMBINED WITH A ChEI | PHARMACOLOGICAL EFFECTS TO BE EXPECTED |
|---|---|
| $M_1$ and $M_3$ partial agonist | Potentiate postsynaptic effects<br>Decrease tolerance & desensitization |
| $M_1$ antagonist | Counteract cholinergic toxicity and desensitization |
| $M_2$ antagonist | Enhance ACh levels and increase its release |

TABLE VI. Combinations of ChEI with muscarinic agonists and antagonists and their effects.

| | Method | Sample Size | Sensitivity (moles) | Reference |
|---|---|---|---|---|
| AChE Activity | microdiver | one cell | $10^{-12}$ | Giacobini and Zajicek, 1956 |
| | gasometric | | | Giacobini, 1957 |
| | radiometric | one cell | $10^{-12}$ | Koslow and Giacobini, 1969 |
| CAT Activity | radiometric | one cell | $10^{-12}$ | Buckley et al., 1967 |
| | | | | McCaman and Hunt, 1965 |
| | | | | McCaman and Dewhurst, 1970 |
| | | homog. | $10^{-12}$ | Goldberg and McCaman, 1973 |
| ACh Level | HPLC-ECD | 10 µl of dialysate | $10^{-14}$ | Cuadra et al., 1994 |
| | | | $10^{-15}$ | Giacobini (1996) |

TABLE VII Cholinergic system: forty years of development of micromethod (1956-1996). AChE: acetylcholinesterase; CAT: cholineacetyltransferase

cognitive impairments observed in AD (Hardy et al., 1985; Decker and McGaugh, 1991). A brain region of particular interest is the frontal cortex because in both humans and rodents it represents the major cholinergic projection of the nucleus basalis magnocellularis (NBM) of the basal forebrain (Mesulam and Geula, 1988). Of the NBM neurons that project to the cerebral cortex, 80-90% are cholinergic in the rat (Rye et al., 1984). Similarly, the major, if not sole, noradrenergic projection to the cortex is the locus coeruleus (LC) (Parnavelas, 1990). Pharmacological alleviation of combined cholinergic NBM/noradrenergic LC lesion-induced memory deficits in rats has been reported (Santucci et al., 1991)

Table VIII compares the effects on ACh, norepinephrine (NE) and dopamine (DA) levels as well as AChE inhibition after systemic administration of six ChEI studied in our laboratory. With the exception of MF-268, not yet tested, they have all shown clinical efficacy. The difference in chemical structure among these compounds is a striking characteristic of new ChEI. Our results show a significant increase in cortex for all three neurotransmitters and for all six ChEIs investigated.

The results reported in Table VIII also suggest that extracellular ACh levels in cortex are not directly related to ChE inhibition, supporting results of previous microdialysis studies showing comparable elevations of ACh levels in spite of different magnitudes of ChE inhibition (Messamore et al., 1993) As a consequence, CNS ChE inhibition can not be considered as a reliable predictor of its effect on concentrations of extracellular ACh in cerebral cortex.

A new aspect of ChEI pharmacology is the effect on neurotransmitters other than ACh (Cuadra et al., 1994). This effect depends not only on dose but also on the type of compound and could be of therapeutic significance.

| Compound | Dose mg/kg | ChE max.% inhib. | ACh | NE | DA |
|---|---|---|---|---|---|
| Physostigmine | 0.3 | 60 | 4000 | 75 | 120 |
| Heptyl-physost. | 2 | 75 | 2500 | 25 | 75 |
| E 2020 | 2 | 35 | 2100 | 100 | 80 |
| MF-268 | 2 | 40 | 2500 | 100 | 60 |
| MDL 73,745 | 2 | 65 | 1020 | 120 | 370 |
| Metrifonate | 80 | 70 | 1700 | 60 | 75 |

TABLE VIII. ChEI effects on ACh, NE, DA levels and ChE activity in rat brain cortex after s.c. administration. E 2020 = (R,S)-1benzyl-4-(5,6 dimethoxy-1-idanon)-2-yl-methylpiperidine (Giacobini et al., 1996); MF-268 = 2, 6-dimethylmorfolin-octyl-carbamoyl eseroline (Zhu et al., 1996); Metrifonate =0,0-dimethyl-(1-hydroxy-2,2,2 trichloroethyl-phosphate) (Mori et al., 1994, 1995b); MDL 73,745=2,2,2-trifluoro-1-(3-trimethylsilylphenyl)ethanone (Zhu et al., 1995)

*Co-administration of ChEI with adrenergic agonists and antagonists demonstrates the interaction between cholinergic and adrenergic systems*

Several studies have indicated close interactions between cholinergic and noradrenergic systems (Decker and McGaugh, 1991). NE decreases the release of ACh from cholinergic terminals in cortex (Vizi, 1980; Moroni et al., 1983). This effect is mediated both directly via alpha-adrenergic receptors on cholinergic terminals and indirectly via NE modulation of gamma aminobutyric acid (GABA) release (Beani et al., 1986). There is also evidence that NE and ACh interact with each other, influencing learning and memory (Santucci et al., 1991). The interaction between ACh and NE appears to be reciprocal as ACh is also able to modulate NE function (Roth et al., 1982; Egan and North, 1985, 1986; Hörtnagl et al., 1987). In a previous study (Cuadra et al., 1994; Giacobini and Cuadra, 1994), we have shown that systemic administration of low doses of PHY and HEP elicit a significant and simultaneous increase in ACh and NE levels. It is possible that the NE elevation seen in our studies could down-regulate ACh levels and decrease the therapeutic effect of these drugs.

*Effect of adrenergic antagonist co-administration*

To investigate this putative cholinergic-adrenergic interaction, we studied the effect of PHY and its analog heptylphysostigmine (HEP) in animals pretreated with idazoxan (IDA), a selective α2-antagonist, on the extracellular levels of ACh, NE, DA and 5-hydroxytryptamine (5-HT serotonin) in cerebral cortex using microdialysis (Cuadra and Giacobini, 1995a).

In this study, we found that IDA administered either systemically or locally into the brain has no effect on extracellular levels of ACh. This suggests NE may not be involved in tonic regulation of cortical cholinergic

activity. The increase of cortical NE release seen after local or systemic IDA administration agrees with the results of L'Heureux et al. (1986) and Dennis et al. (1987). This suggests the effects of IDA on NE release are mediated primarily by α2-adrenoceptors located presynaptically on noradrenergic nerve terminals.

The possibility of further prolonging the effect of ChEI with selective α2-antagonist co-administration and additive DA-ACh interaction may be of therapeutic interest. Specifically, our data suggest that a combination of cholinergic and adrenergic drugs may improve the pharmacological effects of ChEI on several cortical neurotransmitter functions which may represent a significant advantage in AD treatment because of the multiple transmitter deficits seen in the disease.

*Effect of adrenergic agonist co-administration*

In order to obtain further information on cortical neurotransmitter interaction, we evaluated the effect of PHY and its analogue HEP on the extracellular levels of ACh, NE, DA and 5-HT in animals pre-treated with clonidine (CLO), a selective α2-agonist (Cuadra and Giacobini, 1995b).

In agreement with our previous observations (Cuadra et al., 1994; Cuadra and Giacobini, 1995a), which suggested that NE may not be involved in the tonic regulation of cortical cholinergic activity, we detected no effect on extracellular levels of ACh after either systemic or local administration of CLO but NE, DA and 5-HT levels were all decreased. CLO co-administration reduced the effect of PHY on ACh levels, however, HEP administered to animals pre-treated with CLO produced a stronger effect than HEP alone.

The reduction in cortical NE release observed after local or systemic CLO (54% and 57%, respectively) is in agreement with results previously reported by L'Heureux et al. (1986) and Van Veldhuizen et al. (1993). The CLO data, together with our previous results (Cuadra and Giacobini, 1995a) obtained in rats pre-treated with IDA, suggest that ChEI effects on cortical NE release might be mainly mediated by α2-autoreceptors located on noradrenergic nerve terminals (Ong et al., 1991; Coull, 1994).

In analogy, both routes of CLO administration (s.c. and local through the probe) also decreased extracellular levels of DA. This effect of CLO on cortical release of DA might indicate an activation of α2-heteroreceptors localized presynaptically on terminals of dopaminergic neurons which have been demonstrated to modulate its release (Ueda et al., 1983; Dubocovich, 1984). It is well established that DA participates in the control of cognitive function (Brozoski et al., 1979) and plays a role in attention and reward mechanisms (Wise, 1978; Beninger, 1983).

In conclusion, our data suggest that co-administration of a selective α2-agonist such as CLO with ChEI does not represent a favorable pharmacological and therapeutical alternative. Furthermore, the decrease of extracellular DA may represent a negative effect in the treatment of

cognitively impaired AD patients. Considering our previous results with IDA (Cuadra and Giacobini, 1995a), we suggest that a combination of an α2-antagonist with HEP may represent a more favorable approach to improve the clinical efficacy of ChEIs in AD treatment.

*Cholinesterase inhibitors and APP secretion: a possible slowing effect of deterioration?*

The β-amyloid peptide (βA4), one of the major constituent proteins of neuritic plaques in the brain of AD patients, originates from a larger polypeptide denominated amyloid precursor protein (APP) (Kang et al., 1987) APP is widely distributed throughout the mammalian brain including rat brain with a prevalent neuronal localization (Beeson et al., 1994). APP can be processed by several alternative pathways, but the mechanisms responsible for this processing are not completely understood. A secretory pathway is believed to generate non-amyloidogenic soluble derivatives (APPs) following cleavage within the βA4 segment (Sisodia et al., 1990; Esch et al., 1990). Cholinergic agonists regulating processing and secretion of APPs by increasing, as demonstrated *in vitro*, protein kinase C (PKC) activity of target cells (Nitsch et al., 1992, Buxbaum et al., 1992; Nitsch and Growdon, 1994) could decrease potentially amyloidogenic derivatives. We suggested that long-term inhibition of ChE having the effect of increasing the level of synaptic ACh may result in the activation of normal APP processing in AD brain (Giacobini, 1994). This phenomenon could slow down the formation of amyloidogenic APP fragments.

To determine whether ChEI could alter the release of APP we used superfused brain cortical slices of the rat (Mori et al., 1995a) following the method described by Nitsch et al. (1993). Three short- and long-lasting ChEI were tested for their ability to enhance the release of non-amyloidogenic soluble derivatives (APPs) (Mori et al., 1995a). These included: PHY, HEP and DDVP (dichlorvos, a metabolite of metrifonate) at concentrations producing ChE inhibitions ranging from 5% to 95%. All three ChEI elevated

| Drug | Conc (μM) | Increase (% of basal) | ChE Act. (% Inhib.) | APP-KPI mRNA (% of basal) |
|---|---|---|---|---|
| Bethanechol | 1 | 48 | 0 | - |
| | 100 | 53 | 0 | - |
| Physostigmine | .1 | 48 | 25 | - |
| Heptyl-physostygmine | .1 | 41 | 61 | -35* |
| Dichlorvol | .02 | 33 | 95 | - |
| Phorbol myristate | .1 | - | - | +50 |

TABLE IX. Drug-stimulated changes of basal APPs release and APP-KPI mRNA from rat brain (Mori et al., 1995a); *from Giacobini et al., 1995 (5 mg/kg s.c. 48 hrs)

APPs release significantly above control levels (Table IX). Electrical field stimulation significantly increased the release of APPs within 50 min. Similar increase was observed after muscarinic receptor stimulation with bethanechol (BETHA). Tetrodotoxin (TTX) completely blocked the effect of electrical stimulation (Mori et al., 1995a).

The levels of total APP mRNAs in rat cortical slices did not change after incubation with BETHA, DDVP and PHY, but activation of PKC with phorbol 12-myristate-13-acetate (100 nM) increased the level of total APP mRNA by 50% (Table IX) (Giacobini et al., 1995). PHY and MTF administration (0.3 mg/kg and 80 mg/kg s.c., respectively) for 3-48 hrs did not significantly change the levels of APP 695 and APP-KPI (Kunitz-type) protease inhibitor mRNAs (Table IX). HEP administration (5 mg/kg s.c., 3-48 hrs) decreased by 35% the level of APP-KPI mRNA in rat cerebral cortex (Giacobini et al., 1995). AD pathology has been associated with an increase of the KPI-containing forms of APP and the propensity across species to develop neuritic plaques in the cortical regions (Anderson et al., 1989). Our findings suggest that administration of ChEI to AD patients by increasing secretion of APP and inhibiting formation of specific APP mRNAs may exert a neuroprotective effect by activating normal APP processing through a muscarinic mechanism and decreasing amyloid deposition in brain cells.

## CONCLUSIONS

ChEIs, particularly second generation, post-PHY and post-tacrine compounds, affect cortical and presumably sub-cortical neurotransmitters other than ACh. Co-administration of ChEI with adrenergic agonists and antagonists clearly demonstrate a coupling between cholinergic and non-cholinergic systems. This effect depends not only on the dose but also on the type of compound. It might be of additional therapeutical value by activating pathways and circuits other than cholinergic ones which are also hypofunctional in AD. It also represents a possibility of prolonging the effect of ChEI by means of double function hybrid-compounds or co-administration of two drugs. A newly demonstrated *in vitro* feature of ChEI is their ability to enhance the release of non-amyloidogenic soluble derivatives of APP and possibly slow down the formation of β-amyloid deposition in brain. This might slow down cognitive deterioration of the patient treated with ChEI. Recent clinical trials of ChEI extending beyond 36 mo. duration should be able to demonstrate whether or not this pharmacological effect on APP metabolism is of clinical significance. Cholinomimetic alternatives other than ChEI exist and are also being explored pharmacologically and clinically. The most common are based on direct stimulation of muscarinic or nicotinic receptors. However, also with these compounds we suggest combinations of drugs to potentiate the cognitive effect and to decrease side effects.

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# ALZHEIMER DISEASE

## From Molecular Biology to Therapy

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# EXHIBIT 23

# The Cholinergic Hypothesis of Geriatric Memory Dysfunction

Raymond T. Bartus, Reginald L. Dean III
Bernard Beer, Arnold S. Lippa

*Summary.* Biochemical, electrophysiological, and pharmacological evidence supporting a role for cholinergic dysfunction in age-related memory disturbances is critically reviewed. An attempt has been made to identify pseudoissues, resolve certain controversies, and clarify misconceptions that have occurred in the literature. Significant cholinergic dysfunctions occur in the aged and demented central nervous system, relationships between these changes and loss of memory exist, similar memory deficits can be artificially induced by blocking cholinergic mechanisms in young subjects, and under certain tightly controlled conditions reliable memory improvements in aged subjects can be achieved after cholinergic stimulation. Conventional attempts to reduce memory impairments in clinical trials have not been therapeutically successful, however. Possible explanations for these disappointments are given and directions for future laboratory and clinical studies are suggested.

Of the many behavioral impairments identified in the elderly, decreased cognition is generally recognized as one of the most severe and consistent. Controlled laboratory studies indicate that the majority of healthy, elderly persons show reliable declines in cognition in the later phase of life (*1*) and that this disturbance is shared by many other mammalian species, including mice (*2, 3*), rats (*4, 5*), and monkeys (*6–8*). In humans, this problem is often exacerbated by the insidious onset of senile dementia, estimated to affect over 2 million persons in the United States alone, and expected to increase to epidemic proportions during the current decade (*9*). In those cases of senile dementia, the cognitive disturbances often require complete and perpetual institutional care of the patient, compromising the quality of life of the patients and placing emotional and financial burdens on families and society.

Although sociocultural, economic, and psychological factors probably contribute to the cognitive deterioration, the medical community commonly believes that age-related dysfunctions in the central nervous system (CNS) are intimately involved (*10*). Efforts to identify which changes in the CNS play major roles in the cognitive loss have intensified in recent years. Although the specific relationship between age-related CNS dysfunctions and cognitive loss will prove complex, recent evidence suggests that one major factor may be a disruption in the cholinergic neurotransmitter system. This "cholinergic hypothesis" is gaining considerable attention in the geriatric literature and has stimulated clinical trials, which have already attempted to compensate pharmacologically for the presumed cholinergic disturbance. Several paradoxical findings have emerged recently, however, and serious controversies have developed. For this reason, we have attempted to evaluate the available evidence pertinent to this question. We have been guided by three deductive requirements that must be satisfied if the cholinergic hypothesis is to deserve continued attention: (i) specific dysfunctions in cholinergic markers should be found in the brains of subjects suffering from age-related memory loss, (ii) artificial disruption of central cholinergic function in young subjects should induce behavioral impairments that mimic the cognitive loss found naturally in aged subjects, and (iii) appropriately enhancing central cholinergic activity in aged subjects should significantly reduce age-related cognitive deficits. By examining pertinent data from several neurobiological and clinical disciplines within this framework, we have attempted to objectively evaluate the strength of the support for the cholinergic hypothesis. We have also attempted to reconcile certain apparent paradoxes in the literature, identify pseudoissues that have needlessly emerged, and focus on specific critical issues in need of further empirical testing.

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## Evidence for Age-Related Changes in Central Cholinergic Function

Several neurotransmitter systems undergo reliable changes with advanced age (*11, 12*). Although controversy exists regarding which transmitter systems suffer the most dramatic changes with normal aging and whether this pattern differs in the brains of those with Alzheimer's disease, the basic issue crucial to evaluating the cholinergic hypothesis is whether reliable, functionally relevant changes in the central cholinergic system have been identified in aged brain tissue. In most of the research on human cholinergic mechanisms, comparisons have been restricted to Alzheimer's patients and normal age-matched subjects and have excluded young controls. This limitation makes it difficult to determine which qualitative changes in human brain occur normally with age, which may be exacerbated by the insidious onset of senile dementia, and which might be specific to that age-related disease state. Certain generalizations can be formed, however.

One of the more consistent neurochemical findings in the aged human brain is that the activity of choline acetyltransferase (CAT) is markedly reduced in the brains of Alzheimer's patients when compared with age-matched controls (*13*). Because CAT is far from saturated under normal circumstances (*14*), the functional relevance of these decreases in Alzheimer's disease has been questioned. Acetylcholine synthesis in biopsy samples from Alzheimer's patients, however, has been reported to be less than that in samples from age-matched controls (*15*). Furthermore, comparisons between Alzheimer's pa-

tients and age-matched controls revealed a severe loss of neurons in the nucleus basalis of Meynert (located within the substantia innominata) (*16*). Because this brain area is thought to provide the primary cholinergic input to the cortical mantle (*17*), these data offer the possibility that the decrease in cortical CAT in Alzheimer's patients may reflect a specific loss of cholinergic input to the cortex. Further tests are required to determine how characteristic and specific this loss is to patients suffering from senile dementia of the Alzheimer's type. It may be equally important that a positive correlation has been reported between degree of cognitive loss in senile dementia, decreases in CAT activity, and incidence of major neuropathological markers (*18*, *19*).

Although a few studies have reported decreases in CAT activity in brains from nondemented (normal) elderly, many more have failed to find any changes (or found much smaller changes) over a range of disease-free age groups (Table 1). This negative trend suggests that the severe and consistent decrease found in Alzheimer's patients may reflect a disease-specific disturbance.

Although some studies comparing brains from animals of different ages report reliable decreases in CAT activity, these changes are typically small (15 to 25 percent). Further, many studies have failed to find similar decreases (Table 1). Thus, most animal aging data agree with the general human literature, failing to demonstrate large or reliable decreases in the activity of CAT as a function of increased (normal) age (Table 1).

There is no apparent explanation for the success of some authors in finding reliable changes in this enzyme marker with normal aging and the failure of many others. Although differences in assay technique, species, and age of subjects may have contributed to the variability of these results, these variables alone may not adequately explain all the discrepancies reported. Another possibility is that only very small decreases in CAT activity (or number of cholinergic neurons) occur naturally with age and that these changes are difficult to measure consistently. Accordingly, this mild decrease might become greatly exacerbated with senile dementia of the Alzheimer's type, especially in certain brain regions that are particularly vulnerable to the effects of the disease. Also, it seems likely that variations within subregions of certain large brain sites could contribute to differential findings be-

Table 1. Summary of choline acetyltransferase activity (aged rodents compared with young rodents, elderly humans compared with young humans, and Alzheimer's patients compared with age-matched, elderly humans).

| Brain area | Decreased activity: Yes | Decreased activity: No |
|---|---|---|
| | *Aged rodents* | |
| Cortex | Strong *et al.*, 1980 (Rat) (*3*)<br>Unsworth *et al.*, 1980 (*91*) | Timiras and Vernadakis, 1972 (*91*)<br>Meek *et al.*, 1977 (*91*)<br>Reis *et al.*, 1977 (*91*)<br>Strong *et al.*, 1980 (Mouse) (*3*)<br>Ingram *et al.*, 1981 (*91*) |
| Striatum | McGeer *et al.*, 1971 (*91*)<br>Meek *et al.*, 1977 (*91*)<br>Strong *et al.*, 1980 (*3*) | Reis *et al.*, 1977 (*91*) |
| Hippocampus | Vijayan, 1977 (*91*) | Meek *et al.*, 1977 (*91*)<br>Lippa *et al.*, 1980 (*5*)<br>Strong *et al.*, 1980 (*3*)<br>Ingram *et al.*, 1981 (*91*)<br>Sherman *et al.*, 1981 (*20*) |
| Other areas | Unsworth *et al.*, 1980 (*91*) | McGeer *et al.*, 1971 (*91*)<br>Meek *et al.*, 1977 (*91*)<br>Reis *et al.*, 1977 (*91*)<br>Vijayan, 1977 (*91*)<br>Timiras and Vernadakis, 1972 (*91*) |
| | *Elderly humans* | |
| Cortex | McGeer and McGeer, 1975 (*11*)<br>Perry *et al.*, 1977b, 1977c (*91*)<br>Davies, 1978a (*91*)<br>Perry, 1980 (*91*) | Bowen *et al.*, 1976 (*91*)<br>Spillane *et al.*, 1977 (*73*)<br>White *et al.*, 1977 (*91*)<br>Spokes, 1979 (*91*)<br>Carlsson *et al.*, 1980 (*29*)<br>Yates *et al.*, 1980 (*91*) |
| Striatum | | |
| Caudate nucleus | McGeer and McGeer, 1975 (*11*)<br>McGeer and McGeer, 1976 (*91*) | Bowen *et al.*, 1976 (*91*)<br>Perry *et al.*, 1977b, 1977c (*91*)<br>Davies, 1978b (*91*)<br>Carlsson *et al.*, 1980 (*29*)<br>Yates *et al.*, 1980 (*91*) |
| Putamen | McGeer and McGeer, 1975 (*11*)<br>McGeer and McGeer, 1976 (*91*) | Bird and Iverson, 1974 (*91*)<br>McGeer and McGeer, 1975 (*11*)<br>McGeer and McGeer, 1976 (*91*) |
| Hippocampus | Davies, 1978a, 1978b (*91*)<br>Perry *et al.*, 1977b, 1977c (*91*) | Bowen *et al.*, 1976 (*91*)<br>Carlsson *et al.*, 1980 (*29*)<br>McGeer and McGeer, 1975 (*11*)<br>Spokes, 1979 (*91*) |
| Other areas | McGeer and McGeer, 1975 (*11*)<br>McGeer and McGeer, 1976 (*91*) | McGeer and McGeer, 1976 (*91*)<br>Davies, 1978a, (*91*)<br>Davies, 1978b (*91*)<br>Spokes, 1979 (*91*)<br>Carlsson *et al.*, 1980 (*29*)<br>Yates *et al.*, 1980 (*91*) |
| | *Alzheimer's patients* | |
| Cortex, striatum and/or hippocampus: | *Bowen *et al.*, 1976 (*91*)<br>Davies and Maloney, 1976 (*91*)<br>Perry *et al.*, 1977a, 1977c (*91*)<br>*Perry *et al.*, 1977b (*91*)<br>Spillane *et al.*, 1977 (*73*)<br>White *et al.*, 1977 (*91*)<br>Davies, 1978a (*91*)<br>*Davies, 1978b (*91*)<br>Perry *et al.*, 1978 (*18*)<br>Reisine *et al.*, 1978 (*24*)<br>Yates *et al.*, 1979 (*91*)<br>Antuono *et al.*, 1980 (*91*)<br>Bowen and Davison, 1980 (*91*)<br>Carlsson *et al.*, 1980 (*29*)<br>Nordberg *et al.*, 1980 (*91*)<br>†Rossor *et al.*, 1980a (*91*)<br>*Rossor *et al.*, 1980b (*91*)<br>Sims *et al.*, 1980 (*83*)<br>Yates *et al.*, 1980 (*91*)<br>Davies and Feisullin, 1981 (*23*)<br>Davies and Terry, 1981 (*91*)<br>Perry *et al.*, 1981 (*32*)<br>Perry *et al.*, 1981 (*19*)<br>Rossor *et al.*, 1981 (*91*) | |

*Except caudate nucleus. †Except anterior hippocampus and caudate nucleus.

tween investigators. Although these possibilities cannot be objectively evaluated from existing data, future studies carefully specifying tissue origin and location and directly comparing young control subjects with aged subjects and Alzheimer's patients should help resolve this issue. Of course, the question of the functional significance of these subtle (≤ 25 percent) decreases still has to be addressed.

Recent studies in aged animals reveal additional alterations in biochemical measures that suggest presynaptic dysfunctions. Sodium-dependent, high-affinity choline uptake has been reported to be decreased approximately 20 percent under basal conditions in the hippocampus of aged rats (*20*). Under conditions of potassium stimulation, however, choline uptake did not differ in young and aged hippocampus. Further, no age-related differences were observed in either choline or acetylcholine levels. The age-related difference in basal choline uptake was due to changes in the maximum velocity of the enzyme reaction ($V_{max}$) and not in the Michaelis constant ($K_m$). Since the $V_{max}$ for high-affinity uptake is regulated by the activity of cholinergic neurons (*20*), these results suggest a decrease in the activity of septo-hippocampal cholinergic neurons. This possibility has recently received independent corroboration by reports of an age-related decrease in the synthesis of acetylcholine when measured in vivo in two strains of mice (*21*), whereas only marginal decreases were observed in in vitro prisms (*22*) and no loss of synthesis was observed in slices (*20*).

In addition to examining the brains of aged subjects for changes in presynaptic activity, several research groups have investigated postsynaptic muscarinic receptors using radioligand receptor-binding techniques (*23*). Because the majority of the human studies were concerned with changes that might occur specifically with Alzheimer's disease, however, most comparisons were made between brains from Alzheimer's patients and age-matched controls (normal elderly). Although a definitive answer is not yet possible, these studies generally agree that no major difference in receptor binding exists between normal aging and Alzheimer's disease (Table 2). Unfortunately, this comparison cannot address the question of what changes might occur during normal aging. Of the three studies that specifically evaluated changes in muscarinic binding over a range of ages in the nondiseased human brain, two reported significant decreases in binding of muscarinic antagonists in the cortex of the older brains. The receptor densities reported in these elderly subjects were not substantially different from those in Alzheimer's patients, confirming the majority opinion that receptor alterations in the cholinergic system do not occur with Alzheimer's disease to any further extent than that which occurs with natural aging. On the other hand, Reisine *et al.* have reported that the hippocampus of Alzheimer's patients does endure exaggerated loss of muscarinic receptors when compared with that of normal, age-matched controls (*24*). Others have failed to observe this change in the hippocampus (Table 2). The possibility that regional sampling differences within the hippocampus may be responsible for this discrepancy needs to be explored systematically.

Determinations of muscarinic binding have also been performed in aged rodents. Results from these animal studies seem reasonably consistent: of the six reports that have been published, all but one (*3*) reported age-related decreases (20 to 50 percent) in the density of muscarinic receptors with no change in affinity (Table 2). Although perfect agreement does not exist concerning which brain regions exhibit the most reliable changes, the hippocampus, cortex, and striatum have attracted the greatest attention. Once more, the lack of clear definition or identification of what tissue was included when a particular brain site was assayed probably explains many of the apparently contradictory effects in specific brain areas. This problem would seem particularly important when one considers the wide variation in tissue that might be affected when relatively large heterogeneous areas such as cortex and hippocampus are dissected out and the fact that certain regions may be altered by age at different rates. In other words, one major factor for many of the discrepancies in neurochemical changes reported with aging, as well as with dementia, may involve indiscriminate pooling of heterogeneous subregions which exist within classically defined brain sites. Despite these apparent discrepancies, experimental destruction of these same areas in young animals induces specific behavioral deficits similar to many of those found in aged subjects (*25, 26*).

Collectively, there exists good evidence for decreased muscarinic receptor density with normal aging, although little evidence indicates that these changes are more severe in the brains of Alzheimer's patients. This conclusion should not be interpreted to mean that there is no decrease in muscarinic receptors in the brains of Alzheimer's patients. Rather, there appears to be no further loss of muscarinic receptors in Alzheimer's patients beyond that found in age-matched controls. If the decrease in muscarinic receptors is indeed relevant to decreased

Table 2. Summary of muscarinic receptor binding (aged rodents compared with young rodents, elderly humans compared with young humans, and Alzheimer's patients compared with age-matched, elderly humans).

| Brain area | Decrease in receptor density: Yes | Decrease in receptor density: No |
|---|---|---|
| | *Aged rodents* | |
| Cortex | James and Kanungo, 1976 (*92*)<br>Strong *et al.*, 1980 (*3*) | Morin and Wasterlaine, 1980 (*92*) |
| Striatum | Morin and Wasterlaine, 1980 (*92*)<br>Strong *et al.*, 1980 (*3*) | |
| Hippocampus | Lippa *et al.*, 1980 (*5*)<br>Lippa *et al.*, 1981 (*27*) | Morin and Wasterlaine, 1980 (*92*)<br>Strong *et al.*, 1980 (*3*) |
| Other areas | James and Kanungo, 1976 (*92*)<br>Freund, 1980 (*92*)<br>Morin and Wasterlaine, 1980 (*92*) | Morin and Wasterlaine, 1980 (*92*) |
| | *Elderly humans* | |
| Cortex | White *et al.*, 1977 (*91*)<br>Perry, 1980 (*91*) | Davies and Verth, 1978 (*92*) |
| | *Alzheimer's patients* | |
| | *Reisine *et al.*, 1978 (*24*) | †Perry *et al.* 1977 (*91*)<br>White *et al.* 1977 (*91*)<br>Davies, 1978 (*91*)<br>‡Davies and Verth, 1978 (*92*)<br>Perry *et al.*, 1978 (*18*)<br>Reisine *et al.*, 1978 (*24*)<br>‡Antuono *et al.*, 1980 (*91*)<br>Bowen and Davison, 1980 (*91*)<br>†Nordberg *et al.*, 1980 (*91*)<br>*Perry, 1980 (*91*) |

*Hippocampus only. †Including hippocampus.

cholinergic function in normal aging, the persistence of the decrease in Alzheimer's patients must play an equally important role in this disease state.

Although the functional significance of these subtle (and sometimes inconsistent) decreases in receptor density requires further investigation, it has recently been demonstrated that functional disturbances in postsynaptic mechanisms occur in aged animals exhibiting receptor loss and memory impairment (*5*, *27*). This was accomplished by applying microiontophoretic techniques to study responsiveness of hippocampal muscarinic receptors in young and aged Fischer 344 rats. Single-cell recordings revealed that both acetylcholine and glutamic acid iontophoretically applied stimulated pyramidal cell firing rate in proportion to ejection current. However, aged brains became significantly less sensitive to acetylcholine but not to glutamic acid, whereas γ-aminobutyric acid inhibited firing in aged cells slightly more (*27*). This ability of glutamic acid to stimulate cells argues against a generalized age-related decrease in neuronal sensitivity. Rather, these results may be considered direct evidence for a selective impairment of hippocampal cholinergic function in surviving neurons from aged (nonhuman) brains.

It remains to be determined (i) to what extent this decrease in responsiveness to acetylcholine directly reflects the loss of muscarinic receptors, (ii) what other factors (membrane alterations, receptor-effector coupling, and so forth) may also be involved, and (iii) whether they indeed relate to changes in the aging human brain. At the same time, these neurophysiological data, when considered with other neurochemical findings in animals and humans, satisfy an important prerequisite for the cholinergic hypothesis: changes do occur in the cholinergic system with age, and these changes are reflected in decreased functional activity of cholinoreceptive neurons.

Simply demonstrating that age-related changes in the cholinergic system occur does not address the question of whether these changes might be related to the memory loss observed in aged subjects. Age-related changes in the CNS have been observed in many other neurotransmitter systems as well. In certain brain areas, neurochemical markers for other transmitter systems exhibit much more robust changes with normal aging than those reviewed here for the cholinergic system. For example, substantial age-related changes in catecholamines have been reported in the hypothalamus and striatum (*28*). The relationship between these changes and the specific memory loss observed in aged subjects has yet to be addressed systematically. Although some investigators have also reported alterations in catecholamines in Alzheimer's patients (*24*, *29*, *30*), these data have been disputed by other groups and remain controversial (*31*). Finally, certain subpopulations of Alzheimer's patients have been reported to exhibit substantial cell loss in the locus coeruleus (*32*, *33*). Because the locus coeruleus is rich in catecholamine projections to the cortex, differences in degree of locus coeruleus degeneration between undefined subpopulations of Alzheimer's patients might explain the conflicting results regarding catecholamine alterations with senility. However, a recent evaluation of this possibility failed to demonstrate any apparent relationship between changes in cortical activity of dopamine β-hydroxylase and number of locus coeruleus neurons in Alzheimer's patients (*19*, *32*). Further, no correlation was found between loss of dopamine β-hydroxylase activity and the major neuropathological marker (plaque counts) and clinical measures of dementia (*19*). Thus, the role that changes in catecholamines may play in the memory loss of old age and dementia remains uncertain.

One method of gaining additional information about the extent to which changes in various neurochemical systems contribute to the memory loss associated with age and dementia would be to pharmacologically impair function in various neurotransmitter systems in young subjects and compare the changes in memory ability with those occurring naturally in aged subjects. If age-related changes in the cholinergic or any other system contribute to the memory loss observed in old age, pharmacological disruption of that system should induce similar changes in behavior of young subjects.

### Cognitive Effects of Pharmacological Disruption of Cholinergic Function

Deutsch advanced the idea of the role of the cholinergic system in the storage and retrieval of information during new learning (*34*), which has become increasingly accepted. However, the alteration of retention of newly acquired behaviors by pharmacologically manipulating many other neurotransmitter systems (*35*) raises the question of whether the role of the cholinergic system in retention of learned events is any greater than that of other neurotransmitter systems. Moreover, since many of the tasks used in these early studies (such as multiple-trial learning tasks and tests of long-term retrieval) do not display severe age-related deficits (*25*, *36*, *37*), one must question the relevance of these earlier learning and memory studies to the behavioral deficits associated with old age.

More recent studies have directly addressed these issues. Collectively, they provide circumstantial evidence for a role of the cholinergic system in age-related memory deficits. The deficits observed in aged subjects typically occur in situations requiring relatively recent events to be remembered, usually without the benefit of extensive rehearsal or practice (*6*, *7*, *38*). The primary pharmacological data supporting an important cholinergic involvement in this deficit is that blockade of central muscarinic receptors induce a deficit in young subjects which is qualitatively similar to that occurring naturally in aged subjects. In human studies, Drachman *et al.* used a number of clinical measures to find that young subjects tested under a low dose of scopolamine exhibited memory (*39*) and other cognitive (*40*) deficits similar to those found naturally in aged subjects tested on the same clinical battery. The tests which revealed the most severe deficits in both cases involved memory for recent (but not immediate) events.

Aged monkeys tested on a number of different behavioral tasks suffer a very consistent and severe deficit on tasks requiring memory for recent sensory events (*6*, *7*), with greatest deficits under those conditions requiring longest retention of recent information. This deficit shares many conceptual and operational similarities with that suffered by elderly and demented humans (*41*). One of the most consistent and robust pharmacological phenomena observed on this memory task is that young monkeys injected with the central cholinergic receptor blocker scopolamine (but not the peripheral blocker methylscopolamine) exhibited a deficit strikingly similar to that occurring naturally in the aged monkeys (*42*).

Subsequent studies demonstrated that the deficit produced by scopolamine can be partially, but reliably, reduced by the anticholinesterase physostigmine in both humans (*43*) and monkeys (*44*). Similar beneficial effects were not observed with the CNS stimulants methylphenidate (*9*) or amphetamine (*43*). It is therefore unlikely that the retention deficit induced by scopolamine in either human or nonhuman primates can be related to its more general effects on arousal, attention, or similar sedative-like properties.

These data provide additional support

for the possibility that the amnesia induced by scopolamine is due to a specific disruption of cholinergic mechanisms that are important to the behavioral expression of memory. As such, they suggest that an important functional relationship may exist between normal aging, cholinergic malfunctioning, and loss of memory.

In contrast, similar deficits have not been observed with analogous pharmacological blockade of dopamine or β-adrenergic receptors (*45*), supporting the notion that the role of the cholinergic system is somewhat specific. It has been suggested that depletion of dopamine in young monkey frontal cortex by 6-hydroxydopamine induces a cognitive deficit (*46*) qualitatively similar to that observed with aged monkeys (*6*). However, contrary to the effects in aged monkeys and those injected with scopolamine (*42*), the deficit observed with dopamine depletion resembles that found with haloperidol injections (*45*), showing clear deficits on the task but lacking the necessary selectivity on longer delay intervals. Because performance was not differentially affected on long versus short delay intervals, one cannot rule out the possi-

Table 3. Summary of clinical cholinergic precursor studies.

| Study | Dose (g/day) | Substance | Duration | Procedure | Subject population | Effects |
|---|---|---|---|---|---|---|
| Boyd *et al.*, 1977 (*93*) | 5 to 10 | Choline | 2 to 4 weeks | Open | Alzheimer's (70 to 80 years) | No measurable improvement |
| Etienne *et al.*, 1978a (*93*) | 8 | Choline | 4 weeks | Open | Moderate Alzheimer's (76 to 88 years) | One of three possibly improved |
| Signoret *et al.*, 1978 (*93*) | 9 | Choline | 4 weeks | Open | Early Alzheimer's (59 to 78 years) | Claim some improvement, but little data shown |
| Etienne *et al.*, 1978b (*93*) | 25 | Lecithin | 4 weeks | Open | Alzheimer's (42 to 81 years) | No effects on memory scores; three of seven improved on learning rate |
| Smith *et al.*, 1978 (*93*) | 9 | Choline | 2 weeks | Double-blind | Alzheimer's (mean age 77) | No effects on cognitive scores |
| Peters and Levin, 1978 (*64*) | 3.6 | Lecithin | 1 day | Double-blind | Alzheimer's (58 to 79 years) | No effects on memory scores |
| Renvoize and Jerram, 1979 (*93*) | 15 | Choline | 2 months | Double-blind | Alzheimer's (57 to 78 years) | No differences in communication skills |
| Ferris *et al.*, 1979 (*93*) | 12 to 20 | Choline | 4 weeks | Open | Elderly outpatients | No effects on cognitive test scores, including memory |
| Mohs *et al.*, 1979 (*93*) | 16 | Choline | 7 days | Double-blind | Healthy elderly with memory impairment (64 to 86 years) | No effects on any test scores, including memory |
| Whitely *et al.*, 1973 (*93*) | 9 | Choline | 3 weeks | Open | Early Alzheimer's (50 to 58 years) | No effects on cognitive test score; two of eight reported improved on recall test |
| Christie *et al.*, 1979 (*93*) | 2 to 5 | Choline | 9 days | Open | Alzheimer's (53 to 67 years) | No measurable improvement; trend in mild dementia |
| | 28 to 100 | Lecithin | 3 months | Open | Same | No further deterioration after 3 months, compared with patients terminating treatment |
| Mohs *et al.*, 1980 (*93*) | 8 | Choline | 3 weeks | Double-blind | Healthy elderly (62 to 83 years) | No effects on memory scores |
| Fovall *et al.*, 1980 (*93*) | 8 to 16 | Choline | 2 weeks | Double-blind | Early Alzheimer's (55 to 77 years) | Improvement in word recognition only |
| Vroulis *et al.*, 1981 (*93*) | 70 | Lecithin | 2 to 8 weeks | Double-blind | Early-severe Alzheimer's | Improvement in short-term (6 of 15) and long-term (8 of 15) recall and long-term storage (10 of 15). Improvement in EEG frequency (10 of 18) |
| Thal *et al.*, 1981 (*93*) | 4 to 16 | Choline | 2 weeks | Double-blind | Mild to moderate Alzheimer's (49 to 80 years) | No subjective functional improvement nor enhancement of objective cognitive scores, despite doubling of plasma choline concentrations |
| Etienne *et al.*, 1981 (*93*) | 30 | Lecithin | 3 months | Double-blind | Moderate Alzheimer's outpatients (47 to 85 years) | No improvement on any test measures |
| Brinkman *et al.*, 1982 (*93*) | 35 | Lecithin | 2 weeks | Double-blind | Mild to moderate Alzheimer's patients | No improvement in memory |

bility that disturbances in important nonmemory functions (those not directly involved with the storage, maintenance, or retrieval of information in memory), are responsible for the behavioral impairment (*42*, *47*).

Certainly, future research can be expected to identify other neurotransmitter systems playing important roles. In fact, other pharmacological agents (most notably benzodiazepines) can induce similar amnestic performance deficits (*48*). These selective effects are the exception rather than the rule, however, and they emphasize the important role cholinergic mechanisms apparently play in helping to mediate this behavior.

The question of what role the age-related changes in other neurochemical systems, particularly the catecholamines, may play in aged behavior again arises. The high correlation between extrapyramidal Parkinson symptoms and loss of cognitive function (*49*), as well as depression and age, attests (*50*) to the likelihood that these systems are involved in important age-related changes in brain function and behavior. Changes in these systems may also be involved in cognitive dysfunctions related to but different from the memory impairments discussed here. Recent evidence for cell loss in the locus coeruleus with normal aging (*51*) and subgroups of senile patients (*19*, *33*, *52*) supports this possibility. Given that the locus coeruleus provides a major norepinephrine input to the cortex and has independently been associated with performance of learned tasks in rodents (*53*), it is conceivable that age-related declines in locus coeruleus neurons and concomitant catecholamine dysfunction might contribute significantly to the cognitive deterioration of the elderly. To date, however, empirical support is lacking.

Another consequence of age-related changes in catecholamine markers might be to further exacerbate the neurochemical inbalance associated with the cholinergic disturbances, producing greater functional loss. Age-related changes in catecholamines (and other neurotransmitters) could then play a necessary, but not sufficient, role in the memory disorders of the aged. This possibility might explain why pharmacological blockade of these systems fails to induce specific memory impairments similar to those seen in aged subjects and young subjects given central cholinergic blockers.

Although the specific relationship between age-related changes in catecholaminergic function and possible behavioral impairments await further study, an important role for cholinergic dysfunctions in age-related memory deficits has begun to emerge. The human and nonhuman primate studies reviewed corroborate each other and demonstrate that one of the most severe and consistent deficits observed with age occurs on tasks requiring memory for relatively recent events. At the same time, of all the classes of drugs tested on these memory tasks, drugs having anticholinergic effects seem to produce deficits most closely mimicking the natural, age-related memory impairments, satisfying another logical prerequisite for the cholinergic hypothesis. Although more research is needed, particularly concerning the possibility that other neurotransmitter systems may play equally important roles in this impairment, these pharmacological data support a cholinergic role. When these pharmacological data are considered with the correlative neurochemical and neurophysiological changes discussed earlier, this cholinergic interpretation has even greater appeal.

## Facilitation of Geriatric Memory by Cholinomimetics

A question not yet addressed is whether enhancing central cholinergic function can reduce age-related memory deficits. Although neither a necessary nor a sufficient test of the cholinergic hypothesis, studies directed toward this issue may nevertheless provide information useful for determining the overall strength of the evidence for and against the idea. The vast majority of clinical studies concerned with this problem can be classified as one of two types: (i) those attempting to enhance the synthesis and release of acetylcholine by providing abundant amounts of the precursor substances choline or lecithin and (ii) those attempting to enhance cholinergic activity by pharmacological intervention within the synapse or at the receptor site.

The rationale for attempting to improve geriatric cognition with increased amounts of cholinergic precursors is simple. A number of in vitro studies indicate that under certain conditions, increases in brain choline (or lecithin, a normal dietary source of choline) can induce a concomitant increase in the synthesis (and presumably release) of acetylcholine (*54*). Although these findings continue to generate controversy (*55*), recent surveys offer explanations for these discrepancies and conclude that under appropriate conditions (such as increased neuronal stimulation) certain brain regions do increase their rate of acetylcholine synthesis when extra precursor is available (*56*, *57*). Since increased precursor availability may stimulate cholinergic function, cognitive loss might be reduced when abundant quantities of precursor are administered.

Of the 17 studies of either choline or lecithin (Table 3), only one claims substantial improvement (about 60 percent of patients tested). Ten did not obtain facilitative effects on the cognitive tasks (*58*). Although some investigators claim that positive trends seemed to exist in some small subpopulation of the subjects, the effects of the precursors are far from impressive, particularly in well-controlled, double-blind studies (Table 3). The lack of consistent group effects seems particularly striking in view of the wide range of doses tested in these studies and the long-term treatment (of many months) used in many studies. Although it is possible that still undefined subpopulations of patients may benefit from precursor loading, the results to date are disappointing.

The use of cholinomimetic drugs to enhance cholinergic activity as a way of improving geriatric memory has not been as extensive as precursor therapy, but has apparently been somewhat more successful. To date, the most popular cholinomimetic has been the anticholinesterase physostigmine. Early studies with young adults reported moderate improvement on cognitive tests within a very restricted range of single doses (*59*). Doses outside this narrow range produced either no change in performance or marked impairment (*60*). Similar effects have also been reported with young rhesus monkeys (*61*).

Recent studies with physostigmine in aged subjects have also demonstrated reliable facilitation of performance on memory tasks (*61–64*). Contrary to the effects of physostigmine in young subjects, however, the optimal acute dose seems to vary dramatically among individual aged subjects [rhesus monkeys (*61*), Cebus monkeys (*7*), and humans (*63*)]. Although there exist many possible explanations for this phenomenon, the marked improvement on memory tasks achieved with an anticholinesterase is consistent with a cholinergic role in the age-related memory disorders.

In addition to physostigmine, the muscarinic agonist arecoline has been evaluated for effects on performance in memory tasks. After receiving a single injection of arecoline, young adult volunteers exhibited significant improvement in ability to recall recently learned verbal material (*65*). Short-term doses of arecoline can also enhance performance on a

memory task in aged monkeys (*7*) and Alzheimer's patients (*62*). In the monkey study, direct comparisons revealed that the effects of arecoline were more robust and less variable than when the same monkeys were tested under either physostigmine or choline (*66*).

Although additional tests of cholinomimetics in aged subjects (including humans) are needed, it is already apparent that reliable improvement on tasks intended to measure memory can be obtained in the laboratory and clinic by pharmacologically manipulating the cholinergic system. Thus, another important prerequisite of the cholinergic hypothesis has been satisfied. Although the effects observed to date may not be therapeutically outstanding, one must recognize that the ability of physostigmine and arecoline to measurably improve performance must certainly be tempered by the adverse side effects, short half-life, and narrow effective dose range, which are hallmarks of both of these drugs. Further, the specific effects of physostigmine and arecoline on the cholinergic system may not be most consistent with the particular aspects of cholinergic function needed to maximize improvement in cognition. It has been suggested that some other aspect of cholinergic function, or more than a single point in the metabolic pathway, may have to be improved before significant clinical effects are obtained (*67*). Similarly, it may also be necessary to simultaneously improve the function of other undefined systems or affect the balance between the cholinergic and other neurotransmitter systems in order to substantially reduce the behavioral impairments. Presumably, as more is learned about the specific nature of the cholinergic deficiency and its relation to other neurotransmitter systems, drugs with more specific and appropriate actions may be developed, leading to greater therapeutic effects. At the same time, the positive results obtained with current cholinomimetics corroborate the pharmacological, biochemical, and electrophysiological data; together they support an important cholinergic role in age-related memory loss.

These studies have demonstrated that (i) significant changes in cholinergic markers occur in the brains of aged animals and humans; (ii) these changes can be related to a loss of cholinergic function at the neuronal level; (iii) relationships can be established between these changes in the cholinergic system and the loss of memory that occurs with age; (iv) artificial disruption of cholinergic mechanisms in young subjects impairs memory tasks in ways strikingly similar to those that occur naturally in old age and dementia; and (v) a narrow range of doses of certain cholinomimetics can significantly reduce the memory impairments in aged subjects. Although it might be premature to draw any final conclusions from this circumstantial evidence, the data demonstrate that certain logical criteria, prerequisite for accepting the cholinergic hypothesis, have been satisfied and that continued empirical and therapeutic interest is therefore justified.

## Directions for Future Research

A question that is beginning to emerge is why different cholinomimetics seem to produce different results on memory in geriatric subjects. The absence of clear positive effects of choline and lecithin on geriatric patients is also perplexing. Among the many possible explanations, one that is consistent with all available data is that the more directly one stimulates the muscarinic receptor, the more robust and consistent are the effects on memory performance in aged subjects (*7*). Accordingly, even if choline and lecithin increase acetylcholine release, they may have relatively little effect in geriatric subjects because the aged brain may be functionally disturbed at the receptor or coupling mechanism of the cholinoceptive neuron (*5*, *27*, *68*). Such a disturbance might then be most effectively treated by stimulating receptors or the secondary messenger on the effector side of the synapse. Increasing acetylcholine synthesis might do little to alleviate the functional loss since that aspect of cholinergic activity is still relatively intact. Similarly, inhibiting the degradation of acetylcholine released into the synapse may be more effective than that, but still less so than direct agonist stimulation.

Further, drugs that bypass a probable effective link in transmission somewhere beyond the actual binding site might improve performance even more effectively. Research evaluating the effects of different cholinergic agonists and agents in aged humans would be useful, as would that with new classes of drugs to improve cholinergic function in currently unimagined ways.

Other testable possibilities also exist for the inability of choline and lecithin to enhance geriatric memory. One may simply be that peripherally administered precursors do not effectively stimulate cholinergic activity. Although it is becoming accepted that choline has weak muscarinic agonist effects (*69*), its ability to enhance acetylcholine synthesis and release remains controversial (*55–57*). Every study attempting to improve geriatric cognition by precursor loading depends on the validity of this assumption, and thus the data supporting and contradicting this notion must continue to be critically evaluated until a common consensus develops.

Another reason percursors have failed to improve geriatric patients may be that the neurochemical changes are insufficient to produce measurable behavioral effects, particularly on tasks intended to measure memory and other cognitive skills. However, choline induces changes in less complex behaviors in both animals (*70*) and humans (*71*), and there is no a priori reason to expect that the presumed neurochemical factors may be less effective for memory-related tasks. Additionally, a single published account demonstrated increased memory performance when young subjects were administered choline (*72*). Although this question remains open to future experimentation, it seems reasonable that still other factors may be involved.

A third possibility for the apparent paradox may be that the cholinergic dysfunction that contributes to the age-related memory deficit may prevent choline from being effectively converted into acetylcholine in the aged brain. This may be even more true in Alzheimer's disease, where the majority of cholinergic neurons projecting to the cortex (and possibly hippocampus) may be lost, and therefore the machinery to incorporate extra precursor into acetylcholine is no longer intact. Even in the normal aging brain, however, serious deficiencies could impair conversion of plasma choline to intraneuronal acetylcholine. For example, choline uptake (*20*, *73*), choline acetyltransferase activity (Table 1), and oxidative metabolism (*68*, *74*) have all been reported to be decreased in the brains of aged and demented subjects. Since these factors all contribute to normal acetylcholine synthesis, deficiencies in them may not allow choline to be incorporated into acetylcholine as easily as in the brains of younger subjects.

Further, although acetyl coenzyme A (CoA) is normally synthesized de novo in the CNS (*75*), decreases in glucose utilization and oxidative metabolism may decrease the ability of the aged brain to synthesize acetyl CoA, thus making its availability a rate-limiting fac-

tor in acetylcholine synthesis in the aged brain (*21*, *76*). Despite the interest this area of research has recently generated, no studies have directly compared young with aged brain to determine if similar changes in acetylcholine synthesis can be induced with precursor loading, and only one study has evaluated the effects of precursor loading in the aged brain (*77*). Similarly, few systematic studies have yet been performed to determine how the influence of variables such as choline uptake and acetyl CoA may change with age and alter the effects of choline loading (*78*).

These questions raise the possibility that choline is relatively ineffective in stimulating cholinergic activity, particularly when given to aged subjects already suffering deficiencies in the cholinergic system. Although this question needs direct empirical investigation, two recent studies attempted to circumvent problems associated with it while studying the possible beneficial effects of precursor loading.

In the first, the effects of choline were evaluated before the onset of age-related neurobehavioral disturbances occurred (*79*). If age-related changes in the cholinergic system are at least partially responsible for memory impairments, and if dietary manipulation of choline significantly affects cholinergic function, it might be possible to modulate the rate at which memory impairments occur with age by varying the availability of dietary choline. Retired breeder mice (8.5 months old) were placed on purified diets that were either deficient in or enriched with choline. Because life-span tests indicated that reliable deficits in retention of a passive avoidance task are not apparent at this age, it seemed reasonable to assume that the major neurochemical alterations responsible for the deficits were not yet severe in these mice. After 4.5 months the mice were trained on a single-trial passive avoidance task and tested for retention either 24 hours or 120 hours later. Their performance was compared with that of mice of various ages that were maintained on a control diet. Two salient findings were observed: (i) a dramatic decrease in retention of the task was observed in the senescent mice (23 months and older) and (ii) marked differences occurred between the choline-deficient and choline-enriched groups (13 months old). The choline-enriched mice performed as well as 3-month-old mice, whereas the choline-deficient mice performed as poorly as the senescent mice.

This study demonstrated that dietary manipulation of choline can significantly alter behavior in ways that are qualitatively and quantitatively similar to those occurring across the life-span of the mouse. Whether or not these behavioral changes are due to alterations in cholinergic function, per se, remain to be seen. Choline has many important functions in the nervous system, including roles in phospholipid metabolism (*80*). Thus, more general changes in neuronal membranes (or their functions) could have contributed to the deficits. Nevertheless, the data do offer the possibility that certain age-related changes in behavior can be modulated by long-term control of precursor availability.

An important question not yet answered concerns how long into the life-span increased choline will continue to retard the onset of age-related memory losses. These effects represented a retardation in the development of deficits in middle-aged animals. It remains to be seen whether long-term choline administration might reverse existing cognitive impairments in aged subjects (*81*). If the presumed cholinergic dysfunction renders the aged brain relatively incapable of responding to additional precursor stimulation, it might be necessary, with this precursor approach, to intervene before the behavioral impairments and neurochemical dysfunctions fully develop.

Another recent animal study suggests that certain types of pharmacological intervention may potentiate the effects of choline in the aged brain (*67*). This study was based on the possibility that one reason for the lack of significant precursor effects in the geriatric population may be the inability of the aged brain to incorporate or utilize abundant precursor substance. If so, it may be necessary to improve other factors in aged brains before substantial increases in presynaptic cholinergic effects are obtained with precursor loading. For example, although normal cholinergic activity depends on intact oxidative metabolism, several parameters that reflect energy production are decreased in the aged CNS (*82*, *83*). Further, although choline converts into acetylcholine more readily under conditions of increased neuronal activity (*56*), recent circumstantial evidence suggests the activity of certain cholinergic pathways may be reduced in aged subjects (*20*). Thus, either of these (or similar) factors could contribute to a situation in the aged brain that would prohibit extra choline from being effectively utilized for the synthesis of additional acetylcholine and, in turn, would explain the negative results obtained with precursor studies in aged animals and humans.

One way to attempt to compensate for these possible age-related deficits would be to administer abundant amounts of choline while simultaneously giving a drug that might correct other critical age-related neuronal deficiencies. Although no drug yet exists that is recognized as being effective in correcting age-related neuronal dysfunctions, one that is beginning to attract interest for its biochemical and pharmacological properties is piracetam. Several lines of pharmacological evidence indicate that piracetam enables the CNS to function more effectively under hypoxic conditions (*84*) and improves performance in oxygen-deprived (*85*) or aged animals (*86*). Neurochemical determinations suggest piracetam may facilitate conversion of adenosine diphosphate to adenosine triphosphate (*84*, *87*). Other tests indicate that piracetam also enhances intercerebral neuronal activity (*88*) and may deplete hippocampal tissue acetylcholine levels, presumably by increasing release (*89*). Given this profile, piracetam might be able to reduce deficiencies in the aged brain that normally contribute to the lack of significant effects observed with choline loading. This possibility was tested with aged Fischer 344 rats administered saline, choline, piracetam, or combinations of each for 1 week; retention of a one-trial passive avoidance task was measured (*67*).

Aged Fisher 344 rats had previously been shown to suffer severe impairments on this task as a natural consequence of aging (*5*). Control studies suggested that a major source of this impairment is loss of memory for the learned event. For example, control tests demonstrate that possible differences in motor activity or shock threshold cannot explain the age-related differences in the test day (*5*). Further, evaluations of performance after various retention intervals demonstrated that the performance of the aged rats was comparable to that of young rats when tested within a hour after training, but decreased sharply, exhibiting severe deficits within 4 hours after training (*5*). These findings strongly suggest a memory-related component of this age deficit.

The scores of those rat administered only choline did not differ from those of control rats given saline. Although the rats administered piracetam were improved subtly over the saline and choline groups, the retention scores of rats administered the choline-piracetam mixture were several times as high as those

of rats given piracetam alone. These data, therefore, provide preliminary evidence that the effects of increased choline availability in aged animals may be greatly enhanced by the simultaneous administration of a pharmacological agent purported to enhance oxidative metabolism. It is encouraging that a recent clinical trial based on these preliminary animal data found significant improvement in three of ten mild to moderate Alzheimer's patients treated for 1 week with combined choline and piracetam, and all three responders exhibited unusually high choline levels in red blood cells (but not plasma) relative to nonresponders (*90*). Further tests with other drugs to ameliorate other neuronal deficiencies may produce even greater improvement.

It should also be useful to determine mechanisms of action of piracetam and the specific neurochemical changes induced by the combined piracetam-choline treatment. Preliminary neurochemical assays performed on the brains from the behaviorally tested animals revealed modest regionally specific changes in choline and acetylcholine with the combination, the most interesting of which occurred in the hippocampus. Whether these subtle changes were responsible for the more robust behavioral effects remains to be determined (*67*). If certain assumptions of the effects of the drugs are correct, these data suggest that choline may not normally be sufficient to induce measurable behavioral (or neurochemical) improvement in aged subjects, but that correcting other aspects of CNS metabolism may allow this precursor to exert reliable, positive effects in each. The most significant improvement in aged memory may be achieved when multiple, interactive neurochemical dysfunctions in the brain are corrected or when activity in more than one aspect of a deficient metabolic pathway is enhanced. These preliminary data from aged rats suggest that solutions to this problem may not be simple, for different physiological functions may have to be affected; alterations may be necessary at more than one point in the cholinergic or other metabolic pathway, or alternatively, the balance or tone between two or more neurotransmitter systems may need to be improved. Future multidisciplinary studies directed toward identifying the specific alterations responsible for these neurobehavioral dysfunctions should greatly facilitate the search for new and truly effective pharmacological treatment for those aged and demented humans suffering cognitive deterioration.

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# EXHIBIT 24

# Alzheimer's Disease: A Disorder of Cortical Cholinergic Innervation

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*Summary.* Great emphasis is being placed on identification of neurotransmitter systems involved in the symptomatic manifestations of neurological and psychiatric disorders. In the case of Alzheimer's disease, which now seems to be one of the most common causes of mental deterioration in the elderly, compelling evidence has been developed that acetylcholine-releasing neurons, whose cell bodies lie in the basal forebrain, selectively degenerate. These cholinergic neurons provide widespread innervation of the cerebral cortex and related structures and appear to play an important role in cognitive functions, especially memory. These advances reflect a close interaction between experimental and clinical neuroscientists in which information derived from basic neurobiology is rapidly utilized to analyze disorders of the human brain.

One of the most feared and devastating aspects of aging is the deterioration of memory and other mental processes that occurs with increasing frequency in advancing years. About 5 percent or more of the population above the age of 65 years suffers from dementia, a severe impairment in cognitive functions; an additional 10 percent of individuals exhibit mild-to-moderate abnormalities in their cognitive abilities (*1*). Mental infirmity is the major reason for confinement of elderly individuals in nursing homes; and, in the United States, the present cost of nursing home care for patients whose chief symptom is dementia is estimated to exceed $6 billion per year (*2*).

Generally, the onset of senile dementia is heralded by impairments in recent memory. Affected individuals may be able to recall in considerable detail life events from the distant past, but they cannot remember what occurred just minutes earlier (*3*). Inevitably, higher cognitive functions deteriorate and the patients lose the ability to read, write, calculate, or use language appropriately. The loss of cognitive abilities may be accompanied by psychiatric symptoms such as irritability, emotional lability, paranoid delusions, and hallucinations. Affected individuals remain alert until the terminal stages; and the dementia occurs commonly in the absence of focal neurological deficits, such as paralysis or sensory loss, which frequently accompany cerebrovascular disease. Although many individuals remain intellectually adept and lead productive lives into their eighth and ninth decades, it has long been thought that senile dementia is a normal consequence of the aging process.

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## Alzheimer's Disease

Presenile dementia of the Alzheimer's type is a rare disorder in which individuals, typically in their fifth decade, develop a progressive deterioration of cognitive functions clinically indistinguishable from senile dementia. The demonstration that the pathological alterations in the brains of more than half of elderly demented individuals are similar to those found in the brains of patients suffering from the presenile form of Alzheimer's disease (AD) (*4*) suggests that these are related disease processes. In both the presenile and senile forms of the disease, neuropathological examination of the brains disclose characteristic abnormalities (Fig. 1) such as neuritic plaques, which consist of abnormal neurites (principally axon terminals) associated with a core of extracellular amyloid; neurofibrillary tangles, comprised of bundles of paired helical filaments, such as cross-linked polypeptides (*5*), which accumulate within the cell bodies of neurons; and granulovacuolar degeneration, that is, intracellular vacuoles in hippocampal pyramidal neurons. Further evidence that the presenile and senile forms of AD may have a common basis (*6*) comes from genetic studies indicating that the disease may occur as an autosomal dominant in some families. In this article, these two disorders are considered as a single entity, AD.

The cognitive deficits of AD have been attributed to abnormalities in the cerebral cortex and hippocampal formation in that neurofibrillary tangles and senile plaques are prominent in these brain regions. In fact, the density of neuritic plaques in the cortex of AD patients at autopsy correlates with the severity of their cognitive defects (*7*). Since normal aging is associated with a reduction in the number of cortical nerve cells (*8*), it has been difficult to clearly demonstrate that the loss of nerve cells in AD is more severe than in age-matched controls (*9*). However, over the past 10 years, substantial evidence has accrued to indicate that excessive nerve cell loss does occur in the cerebral cortex of AD patients (*10, 11*), with the majority of investigators now affirming that the frontal and temporal cortices are most affected.

## Cholinergic Neurons and Alzheimer's Disease

In that neurotransmitter-specific neuronal systems have been shown to have a role in the pathophysiology of disorders like Parkinson's disease (*12*) and Huntington's disease (*13*), investigators have begun to examine the role of neurotransmitters in the symptoms of disorders of cognition and memory. One strategy used in clinical neuropsychopharmacology is to administer drugs that selectively alter central neurotransmission and then determine whether these manipulations produce symptoms similar to those seen in the disorder.

Drugs that block central acetylcholine (ACh) muscarinic receptors have long been known to disrupt higher cognitive functions and induce transient amnestic states (*14*). When low doses of scopolamine, a centrally active muscarinic receptor blocker, were administered to young adult volunteers, the drug caused selective deficits in recent memory but

did not impair immediate registration or long-term memory (*15*). The scopolamine-treated young adults exhibited a profile on the Wexler adult inventory scale (WAIS) similar to that seen in elderly, drug-free individuals with a significant reduction in performance IQ (intelligence quotient) but not verbal IQ resulting in a comparable "organicity index." The important role played by cholinergic neurons in memory has been substantiated by the findings that drugs which potentiate central cholinergic function enhance recent memory and reverse the performance deficits induced by anticholinergics (*16*). Thus, central cholinergic neurotransmission may play a role in the processing of recent memories, and abnormalities of this system may underlie some of the symptomatic manifestations of AD.

Evidence further implicating the cholinergic system in AD is derived from neurochemical studies of brain tissue obtained from affected patients. Since neurons have highly specialized biochemical processes for the synthesis, storage, and inactivation of their neurotransmitter (Fig. 2), these specialized chemical properties can be used as "markers" for quantifying the innervation of a brain region by transmitter-specific neurons (*17*). The most stable and specific neuronal markers are the enzymes responsible for the synthesis of the neurotransmitter. These enzymes appear to be restricted, in most cases, to the neurons that release the neurotransmitter; their activity remains relatively stable in brain for many hours after death (*13*).

In the cerebral cortex and hippocampal formations of patients who have died with AD, the activity of choline acetyltransferase (CAT), the enzyme that synthesizes ACh, is significantly reduced (by 60 to 90 percent) as compared to age-matched controls that died of unrelated causes (Table 1) (*18–22*). In contrast, muscarinic cholinergic receptors, which are concentrated on neurons receiving cholinergic innervation, have generally not been found to be decreased in the cortex of patients with AD (*20, 23*). Although some reductions in CAT activity have been observed in subcortical structures such as the basal ganglia, these changes are less severe and more variable than those occurring in the hippocampus and cerebral cortex. The activity of acetylcholinesterase (AChE), the enzyme that hydrolyses ACh, was considerably reduced in the cortex and hippocampal formation of patients with AD (*19–22, 24–25*). Although AChE is enriched in cholinergic neurons, it is also present in some nerve cells which do not utilize ACh; for this reason, AChE is not considered a marker specific for cholinergic neurons (*26*).

### Source of Cortical Cholinergic Innervation

Interpretation of the reduction of the markers for the cholinergic neurons in cerebral cortex in AD presented problems because the location of the cell bodies providing cortical innervation was uncertain. The decrements in CAT activity in AD appeared much greater than the degree of neuronal loss in the cerebral cortex (*27*). Nevertheless, a loss of a subpopulation of cortical neurons, which are cholinergic, might not be appreciated with cell counting techniques that cannot distinguish neurotransmitter characteristics of neurons.

Early studies demonstrated that undercutting the cerebral cortex caused a marked reduction in the activity of CAT in the overlying cerebral cortex (*28*). This finding was consistent with the conclusion that cortical cholinergic innervation came from neurons located outside the cortex; however, an alternative explanation, that the enzyme reduction reflected a retrograde degeneration of cortical cholinergic neurons whose axons projected out of cortex, could not be dismissed. Subsequently, Shute and Lewis (*29*), using a histochemical method for AChE, traced the axons stained for this enzyme from the cortex to large neuronal cell bodies in the basal forebrain and concluded that these neurons were the source of cortical cholinergic innervation. However, this interpretation was challenged when noncholinergic neurons, which utilize other neurotransmitters including dopamine and norepinephrine, were also observed to stain intensely for AChE activity (*26*). More recently, immunocytochemical studies have been used to localize CAT-containing neurons innervating the cortex; however, even this method has yielded conflicting results (*30*) which may reflect the exceptional difficulty in purifying this enzyme to homogeneity in order to produce monospecific antiserum (*31*).

A useful approach for identifying the source of transmitter-specific innervation is to ablate discrete regions of the

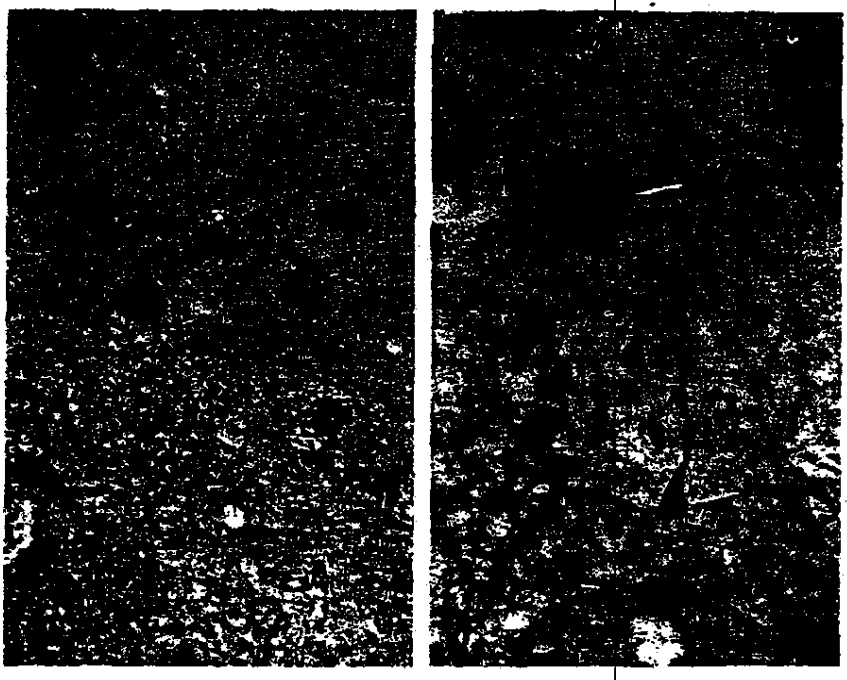

Fig. 1. These photomicrographs, from the brain of an individual with Alzheimer's disease, illustrate, at low (A) and high (B) power, the neuritic plaques and neurofibrillary tangles which are characteristic features of the disorder. This silver impregnation method selectively stains the neurites in plaques and the neurofibrillary tangles in nerve cells, both of which contain abnormal filamentous inclusions. Normal neurons and other elements of the neuropil are not visible in these preparations. (A) The upper layers of the cortex contain several roughly spherical neuritic plaques made up of dark club-shaped processes around a core of amyloid (not stained in this preparation). Several pyramidal neurons contain neurofibrillary tangles impregnated with silver (×40). (B) This micrograph, showing the central part of (A) demonstrates the neurites (axon terminals) forming the corona of the plaque (upper arrow). The cell body and apical dendrite of the layer V pyramidal neuron contain a neurofibrillary tangle (lower arrow); the paired helical filaments are comprised of cross-linked polypeptides, perhaps representing altered neurofilament triplet proteins (×100).

Table 1. Neurotransmitter alterations in the cerebral cortex in Alzheimer's disease. Alterations in the biochemical markers for neurotransmitter specified neuronal systems innervating cortex in AD are summarized. Results were based upon quantitative analysis of markers (enzymes and neurotransmitters) measured in extracts from cortex of postmortem samples obtained from individuals affected with AD and compared to unaffected controls. Extrinsic to cortex refers to the fact that the cell bodies of origin for these neuronal systems are located primarily in the brainstem; intrinsic to cortex indicates that these neurons have their cell bodies primarily within the cortex.

| Neuronal type | Level | References |
|---|---|---|
| Extrinsic to cortex | | |
| Acetylcholine | Marked reductions | *17–21, 28, 29* |
| Norepinephrine | Normal to decreased | *18, 57, 58* |
| Serotonin | Normal to decreased | *57* |
| Intrinsic to cortex | | |
| γ-Aminobutyric acid | Normal to modest decrease | *19, 20, 27* |
| Vasoactive intestinal peptide | Normal | *28, 30* |
| Arginine vasopressin | Normal | *29* |
| Cholecystokinin | Normal | *29, 30* |
| Somatostatin | Marked reductions | *21, 53* |

brain and then examine the consequences of these lesions on neurochemical parameters at target sites. Because developmental studies (*32*) suggested that cortical cholinergic inputs were derived primarily from subcortical regions, neurochemical mapping studies were undertaken in our laboratory to determine the source of cortical cholinergic innervation. To avoid the interpretational problems associated with nonselective, destructive lesions, excitotoxic analogs of glutamate (kainic acid and ibotenic acid) were injected by stereotaxic methods into specific brain regions. Excitotoxins cause a highly selective destruction of neuronal cell bodies in proximity to the injection site but spare axons of passage (*33*). Excitotoxin lesions of the rat ventral globus pallidus (VGP), the site shown by Shute and Lewis (*29*) to contain neurons staining with AChE, caused a marked reduction in cholinergic markers in the ipsilateral cerebral cortex (*34*). Lesions situated in the thalamus, the internal capsule, dorsal globus pallidus, and zone incerta did not reduce cortical cholinergic markers. Significantly, the VGP lesions did not affect the noradrenergic, serotonergic, or histaminergic inputs whose cell bodies are located in the brainstem and whose axons pass through the VGP. Thus, we noted that excitotoxic lesions in the VGP of the rat resulted in selective cortical cholinergic deficits that mimicked those reported in AD (*34*).

After the VGP lesion, subareas of the cerebral cortex, assayed for CAT activity and stained for AChE, showed reductions in enzyme activity and staining to a greatest in the frontal and parietal cortex but negligible in the occipital cortex and hippocampus (*35*). The most extensive lesions of the VGP, which did not affect GABAergic (GABA, γ-aminobutyric acid) markers within the cerebral cortex, were concomitant with a reduction of up to 70 percent of the CAT activity in the frontal and parietal cortex. Accordingly, the cortex must receive a lesser but significant cholinergic innervation from neurons not contained within the VGP. Direct injection of kainic acid into the lateral neocortex caused a major decrease in the activity of glutamic acid decarboxylase (GAD) and only a very modest reduction in CAT. Cortical laminar analysis (*36*), in conjunction with immunocytochemical studies showing neurons containing CAT in cortex (*30*), indicates that there is a small complement of cholinergic neurons intrinsic to cortex; but that the major cortical cholinergic innervation is derived from nerve cells in the basal forebrain (*34–37*).

The magnocellular neurons of the basal forebrain, the primary source of cortical cholinergic innervation in the rat, are among the largest in the brain. These neurons, which stain intensely for AChE, are located in the ventral and medial aspects of the globus pallidus, extend into the hypothalamus, and range rostrally to include the diagonal band of Broca (dbB) and the medial septal nucleus (*38*). Comparative neuroanatomic studies (*39*) indicate that the major part of this cholinergic system in primates is the nucleus basalis of Meynert (nbM) (*40*). Retrograde tracing techniques (*41*) have provided critical information on the topographical organization of these basal forebrain pathways in the monkey (*42*) and rat (*43*). With this anatomical technique, a small amount of the tracer is injected into a discrete region containing axon terminals; the tracer is taken up by the nerve terminals and transported down the axon and back to the neuronal cell body, thus establishing the existence of neuronal connections between these two regions. The neuronal cell bodies in the medial septum and dbB innervate the hippocampal formation and occipital cortex whereas nerve cells in the nbM project primarily to the frontal, prefrontal, and parietal cortex (Fig. 3). Recently, by means of a combination of histochemical staining for AChE to identify the nbM neurons and quantitative assays of CAT in microdissected adjacent sections, the cholinergic nature of the nbM has been confirmed in the primate (*44*).

### Nucleus Basalis in Alzheimer's Disease

The profound reductions in CAT and AChE activities in the cortex and hippocampus of patients dying with AD could result from impaired synthesis of these enzymes, an abnormality of axonal transport of the enzymes from cell bodies to terminals in the cortex, or a degeneration of cholinergic neurons in the basal forebrain. Because several lines of evidence suggested that the primary source of cholinergic innervation to cortex and hippocampal formation was derived from large neurons in the dbB and nbM, we examined these neuronal populations in patients with AD.

The initial case was a 74-year-old man who died after a 14-year history of a progressive loss of memory, impairment of judgment, and deterioration in other cognitive functions. Notably, the patient's father and paternal aunt and uncle suffered from a dementia beginning at approximately 60 years of age (*45*). Histopathological analysis of the brain disclosed neuritic plaques and neurofibrillary tangles diagnostic of AD. Serial histological sections through the forebrain at the level of the anterior commissure were compared with sections from an age-matched control. The patient with AD had a profound and selective loss of neurons within the nbM; whereas nerve cells in the adjacent structures such as the globus pallidus were not affected by the degenerative process.

Because the familial form of AD may represent a separate entity, a subsequent quantitative analysis of neuronal cell loss in the nbM was undertaken in a larger cohort of patients. Five individuals, who suffered from a disorder consistent with AD and who were shown on postmortem examination to have AD, were compared to five similarly aged individuals who had no evidence of dementia (*46*). Nissl-stained sections through the major portion of the nbM were evaluated for the number of neurons in this region. The patients with AD exhibited a highly consistent and marked decrease in neuronal